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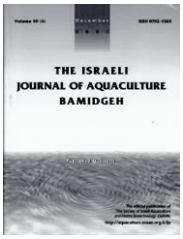

Effects of Different Levels of *Pinus tabulaeformis* Pollen on Growth and Ammonia Stress Resistance of Milkfish Fry (*Chanos chanos*)

Analyn B. Baldove[1], Barry Leonard M. Tumbokon[2], Augusto E. Serrano, Jr.[1,2]*

[1] *Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines*

[2] *National Molecular Biology and Biotechnology, University of the Philippines Visayas, Miagao, Iloilo, Philippines*

Keywords: ammonia-N; stress test; pine pollen; phyto-androgen; ammonia toxicity; ammonia stress resistance

Abstract

The present preliminary study investigated the effects of different levels of dietary pine pollen from *Pinus tabulaeformis* Carr. on the growth and efficiency performance and ammonia stress resistance of milkfish fry (*Chanos chanos*). After acclimation, 600 milkfish fry with average body weight of 0.02 g were stocked in 15, 50L tanks (40 fish per tank). A control diet (without pine pollen, PP) and 4 experimental diets containing various levels of PP, namely, 0, 1.3, 2.6, 3.8, and 5.1 g/kg were prepared and given for 60 days. At termination of the experiment, milkfish were subjected to ammonia stress. Results showed that milkfish fed diets containing various levels of pine pollen exhibited significantly higher weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), and better feed conversion ratio (FCR) than fish fed the control diet. Survival rates of the milkfish during the feeding trial were not significantly different among dietary treatments ($p>0.05$). A separate experiment was conducted in which milkfish were subjected to ammonia stress test following feeding of experimental diets for 2 weeks. Results showed that milkfish fed with diets containing 2.6 to 5.1 g/kg exhibited significantly higher survival ($p<0.05$) than did those fed with the control diet and with the diet containing 1.3 g/kg PP. The optimal inclusion level of pine pollen was estimated using values for SGR and FCR fitted to quadratic model to be 3.0 g/kg. The results of this study indicated that pine pollen could be used as a dietary additive for the young milkfish to enhance growth and immune response to dissolved ammonia.

* Corresponding author. e-mail: aeserrano@up.edu.ph

Introduction

Milkfish, *Chanos chanos*, is an important commodity and one of the most intensively cultured fish species in the Philippines. However, the intensification of its culture has resulted in environmental damage and disease outbreaks that have caused large scale economic losses for fish farmers. Intensive fish culture is associated with chemical, biological, and physical disturbance in the aquatic environment that could alter homeostasis of fish resulting in suppression of the immune system leaving the fish vulnerable to disease. Increased ammonia levels in the water is toxic to fish, and is one result of environmental degradation due to intensive culture (Ruyet et al., 1997).

Pine pollen, the male gamete of the pine tree, is a Chinese traditional medicinal product which has been used as drug and as food for thousands of years. It is a potentially rich source of bioactive compounds capable of boosting both growth and immune response in fish. Masson pine pollen polysaccharides and its esterified product can stimulate the proliferation of spleen lymphocytes and can significantly increase $[Ca^{2+}]_i$ of spleen lymphocytes (Yuan et al., 2011; Hua et al., 2012) in mice, as well as activating T and B lymphocytes isolated from the spleen (Nana et al., 2014; Ming et al., 2014). Pine pollen contains high levels of steroid-like substances such as brassinosteroids that promote rapid growth (Jian et al., 2003) and phytoandrogen that can increase endurance, stress tolerance, sense of well-being, mood, confidence, lean body mass, libido, and sexual response in humans (Velasco et al., 2018). Despite this information, there is a dearth of reports on the use of pine pollen as a feed additive in animal husbandry and in aquaculture. The aim of this study was to investigate the effect of different levels of dietary pine pollen on the growth and stress resistance of milkfish (*Chanos chanos*) where high ammonia concentration was present in the water.

Materials and Methods

Experimental animal and acclimation period

Milkfish fry (ABW= 0.02 g) were procured from Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC-AQD) in Tigbauan Iloilo, Philippines. The fish were stocked and acclimatized in fiberglass tanks provided with continuous aeration at the National Institute of Molecular Biology and Biotechnology Laboratory of the University of the Philippines Visayas, Miagao, Iloilo, Philippines.

Experimental design

For the feeding experiment, a total of 600 milkfish fry were stocked in fifteen 50 L plastic containers at 40 fish per tank in a recirculating system with individual aeration at 20 ppt salinity. They were acclimatized to laboratory conditions and to the control diet for one week. The fish were randomly divided into five groups (four dietary PP treatments and a control group) in a Complete Randomized Design with three replicates per treatment. Each group was fed the experimental diet three times daily (8:00 AM, 11:00 AM and 4:00 PM) 7 days a week to apparent satiation for 60 days. The recirculating system consisted of a physical filter tank (containing gravel and sand), a biological filter containing dead oyster shells and a sedimentation tank with fiber pad filter in a total volume of 1,400 L at a flowing rate of 30 L/h. Water in the system was replaced every 3 days; continuous water aeration was provided and water quality parameters such as water temperature, salinity and pH were monitored two times every day before feeding in the morning and in the afternoon. Nitrite and total ammonia-nitrogen was monitored once a week using commercially available kits. Since milkfish fry were quite small and delicate, sampling was done twice at a 30-day interval i.e. twice for the whole duration of the experiment.

Table 1. Composition of experimental diets containing different dietary levels of pine pollen fed to the milkfish fry (C*hanos chanos*).

Feed Ingredients	*Treatments (% Composition)*				
	A (0.0 PP)	*B (1.3 g/kg PP)*	*C (2.6 g/kg PP)*	*D (3.8 g/kg PP)*	*E (5.1 g/kg PP)*
Peruvian Fish Meal	200.0	200.0	200.0	200.0	200.0
Shrimp Meal	230.0	230.0	230.0	230.0	230.0
Soybean Meal	300.0	300.0	300.0	300.0	300.0
CMC	40.0	40.0	40.0	40.0	40.0
Vitamin Mix	20.0	20.0	20.0	20.0	20.0
Mineral Mix	20.0	20.0	20.0	20.0	20.0
Danish Oil	20.0	20.0	20.0	20.0	20.0
Soybean Oil	20.0	20.0	20.00	20.0	20.0
Corn Starch	144.9	144.9	144.9	144.9	144.9
Cassava Starch	5.1	3.8	2.5	1.3	0.0
Pine pollen	0.0	1.3	2.6	3.8	5.1
Total	1000.0	1000.00	1000.0	1000.0	1000.0
Proximate Composition (% Dry Matter):					
Crude protein	45.49				
Crude fat	5.11				
Crude fiber	3.26				
Ash	13.30				
Moisture	10.84				
NFE	22.00				

Feed preparation

The basal diet composition in the study was composed of Peruvian fish meal, shrimp meal, soybean meal, carboxymethylcellulose (CMC), butylated hydroxytoluene (BHT), lecithin, Danish oil, starch and vitamin mix, and mineral mix; these were purchased from SEAFDEC-AQD in Tigbauan Iloilo, Philippines. All ingredients were passed through 150µm sieve. The composition of the experimental diets is shown in Table 1. The pine pollen was purchased from Zhongshan Yuanhang Co., Zhejiang, China. The different diets were prepared by thoroughly mixing all the pre-weighed dry ingredients before adding the liquid components (i.e. oil and water). Pine pollen was incorporated into the diets at different levels of 0 g/kg, 1.3 g/kg, 2.6 g/kg, 3.8 g/kg, and 5.1 g/kg of feed. The dietary inclusion levels of pine pollen were based on the study by Nian et al (2017) and Adenigba et al. (2017) for Nile tilapia and African catfish. Pine pollen was first dissolved in 95% ethanol before it was added to the pre-mixed feed ingredient. In making the dough, water was added slowly into the mixed dry ingredients. The produced dough was steam-cooked and flattened in trays. The feed then was oven-dried at 60^{o}C for at least 12 h, the dried flakes were cut into smaller sizes, crushed to appropriate size and stored at -4^{0}C until further use.

Growth and feed efficiency parameters

The growth performance and feed utilization efficiency were evaluated using indices such as weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and survival rate. These parameters were estimated as follows:

$$WG\ (g) = FABW - IABW$$

$$FCR = FI\ (g)/WG\ (g)$$

$$PER = (FBW\text{-}IBW)/(FI\ xFP)$$

$$SGR\ (\%\ day\text{-}1) = 100*(lnFABW\text{-}lnIABW)/D$$

$$Survival\ (\%) = 100*Final\ count\ of\ fish/Initial\ count\ of\ fish$$

Where: FABW = final average body weight (g) of individual fish; D = days of culture; IABW = initial average body weight (g) of individual fish; FP= Feed Protein (in decimal); FI = total feed intake of individual fish for the whole duration of the experiment.

Estimation of requirements using quadratic regression

To estimate the maximum response to the dietary pine pollen intake, SGR and FCR values were fitted into a quadratic model (Zeitoun et al., 1976; Chiu et al., 1988). This model is considered to be the most appropriate method to illustrate response parameters with data that are hyperbolic or nearly so, in which peak (or valley in the case of FCR) is reached. This method was used to fit the response data gather from a series of dietary feeding and is expressed in the following equation:

$$R = a + bI + cI^2$$

Where R is the measured response; I is the dietary nutrient concentration; and *a, b,* and *c* are constants that are calculated to provide the best fit of the data. The value of I that produces the maximum response I_{max} is calculated as follows:

$$I_{max} = -0.5*(b/c)$$

Ammonia stress test

For the ammonia stress test, a separate experiment was conducted in which milkfish juveniles (1.5 g average body weight) were fed the same experimental diets for 2 weeks, followed by exposure to ammonia (0.88 mg ammonia-N/L) for 24 h (Wise et al. 1989). 225 milkfish juveniles were used for the stress test at 15 fish/tank, with three replicates for each dietary treatment. The medium was prepared by dissolving the required amount of ammonium chloride (NH4Cl) in seawater at 20 ppt. All tanks were provided with sufficient aeration without feeding. Fish survival was monitored every 15 min during the first hour, every 30 min during the second hour, every hour after the 4th hour, every 4 h after the 16th hour, and every hour on the 24th hour. The fish were considered dead when immobile and showed no response to touch with a glass rod. Cumulative survival was calculated as follows:

*Survival (%) = 100*Initial fish count – dead fish count/initial fish count*

Statistical Analysis

Data on final average body weight weight gain, specific growth rate, and feed conversion ratio and survival of fish exposed to acute ammonia, and high salinity stress test were first tested for homogeneity of variances and normal distribution test before conducting one-way ANOVA. All statistical analyses were conducted using the SPSS v. 16 at 95% confidence level. When significant differences were found, Duncan's Multiple Range test (DMRT) was performed to rank the mean values.

Results

Growth parameters

The growth performance of milkfish fed diets containing different inclusion levels of pine pollen over the 60-day feeding trial is presented in Table 2. WG, SGR, ABW and PER values of milkfish fed with the diet containing 1.3 g of pine pollen/kg diet was not significantly different ($p>0.05$) from those of fish fed with diets containing 2.56 g and 3.84 g/kg (Table 2). All fish fed diets containing PP exhibited higher ABW, WG and SGR values and better FCR value than did fish fed the control diet. Survival of milkfish after 60 days did not differ significantly among treatments ($p>0.05$).

Table 2. Growth performance and survival of milkfish fry (C. chanos) after 60 days of feeding trial.

Pine pollen (g/ kg)	*IABW*	*ABW*	*FI*	*WG*	*SGR*	*FCR*	*PER*	*SR*
0.0	0.02	0.93±0.05^{a}	2.63±0.03	0.91±0.05^{a}	6.56±0.09^{a}	2.89±0.13^{d}	0.77±0.03^{a}	98.33±2.76^{a}
1.3	0.02	1.45±0.11^{c}	2.74±0.05	1.43±0.11^{c}	7.31±0.13^{c}	1.94±0.13^{a}	1.15±0.08^{c}	97.50±3.5^{a}
2.6	0.02	*1.27±0.01^{bc}*	*2.66±0.01*	1.25±0.01^{bc}	7.09±0.01^{bc}	2.13±0.01^{abc}	1.04±0.01^{bc}	100.00±0.00^{a}
3.8	0.02	1.37±0.08^{c}	2.71±0.05	1.35±0.08^{c}	7.22±0.09^{c}	2.02±0.08^{abc}	1.10±0.04^{bc}	97.50±3.57^{a}
5.1	0.02	1.15±0.02^{b}	2.62±0.03	1.13±0.02^{b}	6.92±0.03^{b}	2.32±0.04^{c}	0.96±0.02^{b}	99.17±2.76^{a}

Initial average body weight (IABW), Average body weight (ABW), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency r individual feed intake (FI)ratio (PER) and survival rate (SR) of L. vannamei after the 60-day feeding experiment.

All values are expressed as mean±SEM(Standard Error of the Mean).

Means in the same column sharing the same superscript are not significantly different (P>0.05)

Ammonia Stress test

The survival rate of milkfish fed diets containing different levels of pine pollen after being subjected to high ammonia stress test for 24 h are shown in Table 3. Survival rates of milkfish fed with diets containing 2.6 to 5.1 g/kg PP were significantly ($p<0.05$) higher than those fed with the control diet and the diet containing 1.3 g/kg PP.

Table 3. Survival of milkfish (*Chanos chanos*) fry after 24 h exposure to acute ammonia

Pine pollen (g/kg)	*Survival (%)*
0.0	13.3 ± 5.4^{ab}
1.3	8.9 ± 7.5^{a}
2.6	46.7 ± 12.1^{bc}
3.8	45.6 ± 3.9^{bc}
5.1	64.4 ± 4.9^{c}

All values are expressed as mean±SEM.Means in the same column sharing the same subscript are not significantly different (p>0.05).

Estimation of optimum level of pine pollen

The SGR and FCR values of milkfish fed different levels of pine pollen were fitted to a quadratic model to estimate the optimum level of inclusion (Figure 1). The optimal inclusion levels of pine pollen for SGR and FCR were estimated to be 2.9 g/kg and 3.0 g/kg, respectively.

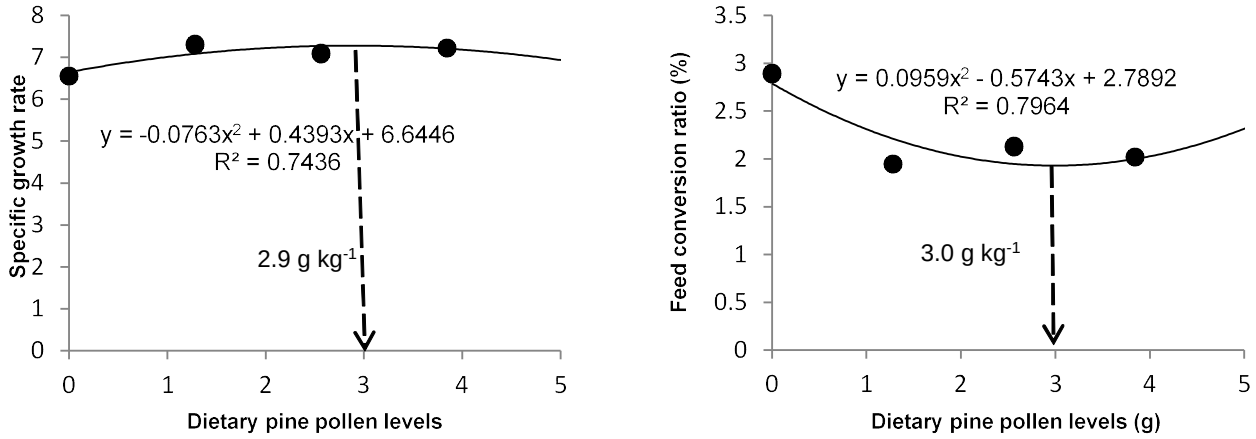

Figure 1. SGR and FCR data fitted to a quadratic model to determine the optimum inclusion level of pine pollen in the diet milkfish (*Chanos chanos*).

Discussion

The present study was performed to examine pine pollen as an alternative to synthetic growth promoters in fish culture. To the best of our knowledge, this is the first study to investigate the effects of pine pollen on the growth and stress resistance of milkfish fry to lethal/sublethal dosage of ammonia. The results of the present study showed that the growth and efficiency of milkfish was positively affected by the dietary pine pollen at various levels under laboratory conditions. Milkfish fed diets containing pine pollen showed better growth performance in terms of WG, SGR, PER, and better FCR than those fed the control diet. This could be attributed to the anabolic and androgenic effects of androgen from the pollen. The results of the present study corresponded with those obtained in *Clarias gariepinus* (Adenigba et al., 2017) and in *Oreochromis niloticus* (Nian et al., 2017). In addition, in *Clarias gariepinus* fed with diets containing phytoandrogen and androstenedionen (occurring naturally in pollens from certain pine trees and European cactuses), improved growth performance of the African catfish in terms of

weight gain, PER and FCR was observed after 120 days of feeding (Turan and Akyurt, 2005).

In the present study it appeared that the anabolic effect of androgen from pine pollen was best achieved in milkfish treated with an intermediate dose of 3.0 g/kg. The high survival rate in the treated fish during the feeding trial in the present study indicated that the treatments used did not adversely affect the general health of the milkfish. These results concur with observations in *Clarias gariepinus* and *Oroechromis niloticus* (Adenigba et al., 2017; Nian et al., 2017).

Feeding milkfish with diets containing a small amount of pine pollen resulted in a higher ammonia tolerance than did those fed the control diet containing no pine pollen. The higher survival of milkfish fry fed dietary PP from 2.6-5.1 g/kg were relatively high with a range of 45.6% to 64.4% after 24 h of ammonia exposure to 0.88 mg/L ammonia-N. The enhancement of immune response of milkfish to acute ammonia stress test was probably due to antioxidant and polyphenolic contents of the pine pollen. Moreover, it has been already found that pine pollen and pine pollen derivatives could eliminate reactive oxygen species in rodents and could be considered as a source of antioxidant agents (Lee et al., 2009; Yue and Yu 2013). In addition, feeding tilapia with a diet supplemented with pine pollen induced immunity of the fish producing specific antibodies against naturally infecting *Aeromonas hydrophila* within the fish body system (Baluran et al., 2018). The results of the present study indicated that diets supplemented with pine pollen could have enhanced non-specific immune response of the milkfish. There have been no previous reports on the effect of pine pollen on the response of milkfish to environmental stresses such as ammonia.

In conclusion, dietary supplementation of pine pollen significantly enhanced the growth performance of milkfish fry at the inclusion level of 3.0 g/kg diet of pine pollen and enhanced stress resistance of milkfish fry to acute ammonia. The results of this study suggest that pine pollen could be a potential source of a natural, cheap, and convenient immunostimulant that could help improve fish welfare. The present study provided new information on the use of pine pollen in aquaculture which could encourage further research on this topic.

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References

Adenigba, I., Tumbokon, B.L., Serrano Jr., A.E., 2017. Androgenic and Anabolic Effects of Pinus tabulaeformis Carr. Pollen in Clarias gariepinus. *Isr. J. Aquacult.-Bamidgeh, AquacultureHub,* IJA_69.2017.1388.

Baluran, S. M., Quiazon, K. M., Garcia, G., Fernando, S. I., Velasco, R., 2018. Immunostimulatory effect of Benguet (*Pinus kesiya*) pollen on Nile tilapia (Oreochromis niloticus L). *Int J Biol, Pharmacy and Allied Sci* 7(9): 1652-1663.

Chiu Y.N., Austic R.E., Rumsey G.L., 1988. Effect of feeding level and dietary electrolytes on the arginine requirement of rainbow trout (Salmo gairdneri). *Aquaculture* 69:79-91.

Hua, M., Huili, C., Yueran, L., Rui, L., Yue, G., 2012. Influences of sulfated polysaccharide from pine (*Pinus massoniana*) pollen on the regulation of [Ca2+]i in splenocytes. *Chin. Pharmacol. Bull*., 28: 1460-1464.

Jian, J., Wu, Z., 2003. Effects of Traditional Chinese Medicine on Non Specific Immunity and Disease Resistance of Large Yellow Croaker, Pseudoscia enacrocea (Richardson). *Aquaculture* 218 (1–4):1–9.

Lee, K.H., Choi, E.M., 2009., "Effect of pine pollen extract on experimental chronic arthritis," *Phytotherapy Res*,. 23(5): 651–657.

Ming, L., Nana, L., Yue, G., 2014. Influences of sulfated polysaccharide from pine (*Pinus massoniana*) pollen on the immunomodulatory effects of B lymphocytes in mice *Chin. J. Cell Biol.* 4: 461-469.
Nana, L., Ming, L., Yue, G., 2014. Immunomodulatory effects of sulfated polysaccharide and its esters from pine pollen on mice T lymphocytes. Chin. Pharmacol. Bull. 8:1151-1155.
Nian, C., Tumbokon, B.L., Serrano Jr., A.E., 2017. *Pinus tabulaeformis* pollen as replacement for 17-alphamethyltestosterone in the diet of *Oreochromis niloticus* larvae for sex reversal and growth. 69.2017.1399.
Ruyet, J.P-L., Galland, R., Le Roux, A., Chartois, H., 1997. Chronic ammonia toxicity in juvenile turbot(Scophthalmus maximus). Aquaculture 154 (1997) 155-171
Turan, F., Akyurt, I., 2005. Effects of androstenedione, a phytoandrogen, on growth and body composition in the African catfish (Clarias gariepinus). *Isr. J. Aquacult.-Bamidgeh,AquacultureHub,* 57: 62–66.
Velasco, R.R., Dollente, D.J.M., Natividad, L.R., Abella, T.A., 2018. Benguet pine pollen as (Pinus kesiya) as natural source of phytoandrogen. International Journal of Biology, *Pharmacy and Allied Sciences,* 7(6): 1121-1132 doi.org/10.31032/IJBPAS/2018/7.6.4472.
Wise, D.J., Weirich, CR., Tomasso, J.R., 1989. Toxicity of ammonia to red drum Sciaenops ocellatus fingerlings with information on uptake and depuration. *J. World Aquacult. Soc*. 20, 188-192.
Yuan, L., Hong, Z., Yun, C., Yu, X., Yue, G., 2011. Effects of polysaccharide from masson pine pollen and its sulfated derivative on the cytosolic $[Ca^{2+}]_i$ in mice splenocytes *Pharm. Biotechnol*., 18: 396-398.
Yue, G., Yu, X., 2013. Effects of pine pollen polysaccharide and its sulfate on the production of ROS in cardiomyocytes. *J Med Plant Res*. 7(25): 1818-1824. DOI 10.5897/JMPR11.1312.
Zeitoun I.H., Ullrey D.E., Magee W.T., Gill J.L., Bergen W.G., 1976. Quantifying nutrient requirements of fish. *J Fish Res Board of Canada* 33(1):167-172.